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(54) Title: SOLID PHASE ASSEMBLY AND RECONSTRUCTION OF BIOPOLYMERS

(57) Abstract

The present invention concerns the construction of solid phase assembly of biopolymers through assembly of shorter biopolymer sequences, for example, assembly of genes from oligonucleotides, polypeptides from oligopeptides, and polysaccharides from oligosaccharides. The present invention also relates to the remodeling, or reconstruction of biopolymers, wherein a section of the biopolymer sequence is excised, then replaced by a modified segment.

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SOLID PHASE ASSEMBLY AND RECONSTRUCTION
OF BIOPOLYMERS

5

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns the construction of biopolymers through assembly of shorter biopolymer sequences, for example, assembly of genes from oligonucleotides, polypeptides from oligopeptides, and polysaccharides from oligosaccharides. The present invention also relates to the remodeling, or reconstruction of biopolymers, wherein a section of the biopolymer sequence is excised, then replaced by a modified segment.

Background of the Invention

DNA is the chemical substance that makes up the genomes of most life forms. Two properties of DNA that are fundamental to its in vivo function and to the ability of scientists to manipulate it in vitro are that (i) DNA is composed of four different subunits ("bases"), adenine (A), guanine (G), cytosine (C) and thymine (T), linked together by a sugar-phosphate backbone to form long polymeric strands, and (ii) two "complementary" strands of DNA come together to form a double helical DNA molecule by specific hydrogen bonded base pairing (A pairs with T and G pairs with C). This specific base pairing plays an important role in chromosomal replication, a process in which the two DNA strands of a chromosome become separated, then a DNA polymerase enzyme uses each strand as a "template" to synthesize a complementary strand which then base pairs with the template strand, thereby resulting in the formation of two chromosomes from one.

1 Base pairing also functions in the process of
"transcription," wherein an RNA polymerase enzyme utilizes
the base pairing properties of one strand of a gene to
5 synthesize a "messenger RNA" molecule (a nucleic acid in
which uracil replaces thymine and the sugar is ribose
instead of 2"-deoxyribose). The messenger RNA is
subsequently "translated" into protein as directed by the
genetic code (each 3-base '"codon" in the messenger RNA
specifies a certain amino acid to be incorporated into a
10 protein product). Thus, for each gene, the coupled
transcription/translation process results in biosynthesis
of a protein molecule that contains an amino acid sequence
that is encoded by the base sequence in the DNA. In turn,
the amino acid sequence in the protein determines how the
15 protein folds into a specific structure and how it
interacts with other molecules in its biochemical
function, for example, catalysis of a specific chemical
reaction in the case of an enzyme.

20 Finally, base pairing forms the basis of the
"annealing" reaction that is employed in a variety of
laboratory DNA manipulations: Two separated DNA strands
will spontaneously pair up to form a duplex structure
throughout the region(s) of complementarity if, and only
if, they contain one or more stretches of complementary
25 base sequence.

Recent development of techniques for manipulation
of genes and proteins, which are used extensively in the
fields of genetic engineering and biotechnology has
allowed chemical synthesis of DNA molecules of any desired
30 base sequence, which can be used to alter existing genes
or create new ones. This capability, a fundamental tool
in "protein engineering," allows analysis of the
structure/function relationships within proteins and
creation of superior enzymes and drugs in the
35 biotechnology and pharmaceutical industries.

1 Over the past thirty years, methods for chemical
synthesis of DNA have rapidly developed. Michelson and
Todd first chemically synthesized a dinucleotide
containing the correct phosphodiester linkage (Michelson
5 and Todd, J. Chem. Soc.:2632-2638 (1955)). Khorana
developed and used the phosphate triester method of DNA
synthesis to produce a gene encoding transfer RNA
(Agarwal, et al., Nature (Lond.), 227:27-40, (1970)).
More efficient phosphate triester DNA synthesis procedures
10 were subsequently developed (Letsinger and Ogilvie, J. Am.
Chem. Soc., 89:4801-4803, (1967); Narong et al., Meth.
Enzymol., 65:610-620 (Letsinger et al., J. Am. Chem. Soc.,
97:3278-3279 (1975); Beaucage and Caruthers, Tet. Lett.,
22:1859-1862, (1981)). Efficient utilization of solid
15 phase supports for chemical synthesis of DNA have been
disclosed (Matteucci and Caruthers, J. Am. Chem. Soc.,
103:3185-3191 (1981); Sproat and Bannwarth, Tet. Lett.,
24:5771 -5774 (1983)).

20 The use of solid phase supports for chemical
synthesis of DNA contributed most importantly to the
ability to rapidly and efficiently synthesize DNA
chemically, because the growing chain is covalently
attached to an insoluble support, permitting reagents to
be washed away between chemical steps, thus eliminating
25 the need to purify the polynucleotide product after each
addition of monomer. Furthermore, solid phase synthesis
permits automation of the process, so that each base
addition (via multistep reaction cycle) can be carried out
in about ten minutes at room temperature (Smith, American
30 Biotechnology Laboratory (Dec., 1983); Caruthers, Science,
230:281-285 (1985)).

35 It is now possible to construct a duplex DNA
molecule encoding a protein or portion thereof, and use of
the synthetic duplex fragment to construct a recombinant
DNA which can be expressed in vivo to obtain a novel gene
product. However, the widespread application of gene

1 synthesis has been hindered by: (i) the high cost of
synthesis of all the oligonucleotides needed to assemble
an average gene (typically \$5,000 to \$20,000); and (ii)
the slow and labor intensive nature of gene assembly from
5 synthetic oligonucleotides. Chemical synthesis of DNA
currently produces polynucleotides up to 100-150 bases in
length (and at the upper limits the yield is very low).
The coding portion of the average gene, however, consists
of 1000-base pairs. Thus, in order to assemble a gene, a
10 series of overlapping, complementary oligonucleotides must
be synthesized, then "annealed" together (i.e., mixed
together and incubated under conditions that favor
formation of the double helix between complementary
sequences within the two strands). The duplex DNA, which
15 contains strand interruptions at alternating positions
along the two strands, is then converted to a contiguous
duplex segment, by enzymatic ligation. Only then can the
duplex DNA be cloned into a vector for subsequent analysis
and expression (protein production). In practice, the
20 correct assembly of a gene from a complex mixture of
oligonucleotides is difficult to achieve in a single
annealing step, due to formation of a variety of
undesirable annealing products. A series of laborious
purification and analytical steps must normally be carried
25 out before the intact gene is isolated.

Solid phase procedures for chemical synthesis of
peptides are frequently based on the protocol of
Merrifield, which has been successfully used for synthesis
of enzymatically active, 124-residue ribonuclease A (Gutte
30 and Merrifield, J. Biol. Chem., 246:1922-1941 (1971)).
This procedure uses polystyrene-divinylbenzene supports,
t-butyloxycarbonyl (tboc) amino group protection, and
DCC-activated condensation with symmetric anhydride
intermediates, and has been adapted for fully automated
35 peptide synthesis. Another procedure for chemical
synthesis of peptides (known as the "Fmoc" procedure)

utilizes a composite polyamide-Kieselguhr support (superior for continuous flow synthesis), together with fluorenylemethoxycarbonyl (Fmoc) amino group protection, and N-hydroxybenzotriazole-activated condensation with pentafluorophenyl ester (PFPE) intermediates or symmetrical anhydride intermediates (Auffret and Meade, Synthetic Peptides in Biology and Medicine, Alitalo et al. (Eds.), Elsevier Science Publishers, Amsterdam, (1985)).

As with DNA, chemical synthesis of peptides, prior to the present invention was possible for chain lengths up to 100-200 residues (with very low yields at these upper limits). More typically, peptides of 20-30 residues are produced. Assembly of peptides into large polypeptides is technically feasible, by ordered, stepwise condensation of peptides via the Fmoc procedure. But again, this approach is expensive and requires laborious, time consuming purification of products after each block condensation reaction.

The high expense of synthesizing the large numbers of polynucleotides and peptides needed to assemble genes and proteins is largely overcome by use of the segmented synthesis technology described in U.S. patent application Ser. No. 07/000,716, filed Jan 6, 1987), whereas the assembly of these biopolymer fragments into genes and proteins remains cumbersome and time consuming.

Another technology in genetic engineering and biotechnology is the use of enzymes to manipulate the genetic material in recombinant DNA research. Restriction endonucleases (enzymes that recognize and cleave DNA at specific sequences, 4-8 base pairs in length) are used to isolate specific regions of a chromosome, and DNA ligases (enzymes which join together fragments of DNA resulting from action of restriction enzymes) are used to "clone" the specific DNA fragments into extrachromosomal replicating genomes (plasmids or viral DNAs), known as "vectors" Berg, Science 213:296-303 (1981). The resulting

1 recombinant DNA is used to analyze the base sequence of a
cloned fragment, or to produce large amounts of a protein
coded for by a cloned gene. As discussed above, a
powerful extension of this technology is the use of
5 chemically synthesized duplex DNA fragments in place of a
naturally occurring "restriction fragment" in formation of
a recombinant DNA.

10 Although recombinant DNA technology represents a
powerful tool in molecular biology research and genetic
engineering, the labor intensive purification steps and/or
analysis of numerous reaction products are required before
a desired recombinant DNA product can be isolated.

15 Direct manipulation of proteins analogous to
recombinant DNA methods (Offord, Protein Engineering,
1:151-157, (1987)), allows use of specific endopeptidases
to excise specific segments from proteins, and then to
replace these by synthetic pieces, chemically different
from the natural peptides. This "recombinant protein
technology" also requires laborious purification steps and
20 analysis of different reaction products in order to
isolate the desired engineered protein.

25 Thus, despite the tremendous power of currently
available genetic engineering techniques, further
improvements are needed in the speed, efficiency and
economy of biopolymer manipulations. Accordingly, due to
the shortcomings of the present procedures, there exists a
need for a process for rapid, low cost, efficient and
accurate assembly of biopolymers from their subcomponents,
and for rapid and convenient in vitro remodeling of
30 biopolymer sequences, whereby isolation of the desired
engineered biopolymer is achieved with a minimum of
purification and analytical steps.

SUMMARY OF THE INVENTION

35

It is therefore an object of the present
invention to provide an improved process for assembly of

1 biopolymers from subcomponents thereof.

Another object of the present invention is to provide a process for more rapid assembly of genes or gene segments by stepwise annealing of synthetic
5 oligonucleotides.

Yet another object of the present invention is to provide a more cost effective process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides requiring less labor and materials.
10

A further object of the present invention is to provide a more efficient process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides requiring fewer purification and analytical steps.
15

Still another object of the present invention is to provide a more efficient process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides, providing a greater yield of the desired end-product.
20

An additional object of the present invention is to provide a faster, more efficient and less costly process for assembly of peptides into polypeptides.
25

A further object of the present invention is to provide an improved process for replacement of a specific segment of a DNA molecule by an analogous, modified segment or by a different segment.
30

Yet another object of the present invention is to provide an improved process for replacement of a specific segment of a polypeptide molecule by an analogous, modified segment or by a new, unrelated segment.
35

Still another object of the present invention is to provide an improved process for deletion of one or more specific segments within a nucleic acid or protein.

An additional object of the present invention is to provide an improved process for insertion of one or more oligomeric segments at specific locations in a nucleic acid or protein.
40

1 Thus, in accomplishing the foregoing objects,
there is provided in accordance with one aspect of the
present invention, an improved, general procedure for
construction of biopolymers, comprising the following
5 steps: (1) attachment of a biopolymer subcomponent to a
solid phase support; (2) attachment of the next biopolymer
sequence to one end of the support-bound component; (3)
washing away of excess, unattached biopolymer sequences
added in step (2); (4) ordered, stepwise attachment of
10 oligomeric biopolymer sequences to the free end of the
support-bound component (by repeated conduct of steps (2)
and (3)), resulting in assembly of the biopolymer; and (5)
release on the assembled biopolymer from the support. All
15 steps in the foregoing process can be carried out in a
suspension of the support, or alternatively, in a packed
bed column fitted with porous means at both ends to
provide a flow-through system. The biopolymer to be
constructed by this process is chosen from among the group
consisting of DNA (genes or gene segments), polypeptides
20 (proteins), polysaccharides, or any other biopolymer
composed of subsections that can be joined together. The
"starting" biopolymer component initially attached to the
support can range widely in length, for example 1-100
residues, the precise length being a matter of choice, but
25 the support-bound starting component will typically be
10-50 residues in length. The nature of the solid phase
support is a matter of choice, provided that the structure
of the support does not sterically hinder the assembly of
the desired high molecular weight biopolymer. The linkage
30 of "starting" biopolymer component to the solid phase
support is a matter of choice, readily achievable by one
skilled in the art, using a variety of prior art methods.
The nature of the stepwise linkage of oligomeric
biopolymer segments during the assembly process, as well
35 as the method of cleavage of final product from the
support, will depend on the type of biopolymer being

1 constructed, details of which are given in the embodiments
described below.

5 In accordance with one specific aspect of the present invention, there is provided an improved process
for assembly of a gene (or gene fragment) from synthetic oligonucleotides, comprising the following steps: (1) attachment of a "starting" oligonucleotide to the solid phase support, at or near one of its two ends; (2) addition of a molar excess of the next oligonucleotide in
10 the gene to be assembled, one end of the added oligonucleotide being complementary in base sequence to the free end of the support-bound oligomer, to form a molecule in which one end of the added oligonucleotide is base paired with the support-bound oligomer, leaving a
15 single-stranded tail at the other end of the added oligomer; (3) washing away of the unannealed free oligonucleotides; (4) repeated cycles of oligonucleotide addition/annealing/washing, carried out until the desired gene or gene fragment has been assembled; and (5) release
20 of the assembled DNA from the support.

In step (1) of this preferred embodiment, the solid phase support is first derivatized with a nucleoside, then the "starting" oligonucleotide is synthesized on the solid phase support, using standard phosphate triester or phosphite triester procedures, the linkage of this synthesized oligonucleotide to the support being retained and utilized in the subsequent gene assembly. The solid phase support in this embodiment is preferably nonporous glass beads of small diameter (5-50 micrometers) or small diameter (5-50 micrometers) glass beads containing large diameter (1000-5000 Å) pores. Derivatization of the glass beads with nucleoside can be achieved by a variety of prior art methods that are readily apparent to one skilled in the art. For example, the 3"-urethane linkage of a nucleoside to the glass via long chain alkylamine spacer arm (Sproat and Brown, Nucl.

1 Acids Res., 13:2979-2987, (1985)) can be employed to yield
a solid phase support suitable for synthesis of the
"starting" oligonucleotide by standard phosphoramidite or
phosphate triester methods. The urethane linkage is
5 largely retained during the deprotection of exocyclic
amino groups, and can subsequently be utilized for solid
phase gene assembly. Alternatively, solid phase synthesis
of the "starting" oligonucleotide can proceed via the
phosphoramidite method or phosphate triester method on
10 glass beads derivatized with nucleoside via the standard
3"-O-succinyl linkage, provided that the sequence of the
"starting" oligonucleotide is chosen to avoid nucleoside
residues containing exocyclic amino groups, since the
alkaline condition normally required for deprotection of
15 exocyclic amino groups would cleave the DNA from the
support. For employment of the O-succinyl linkage a
starting oligonucleotide sequence consisting of thymidine
and inosine residues would be appropriate.

In step (1) of another preferred embodiment
20 (attachment of the "starting" oligonucleotide to the solid
phase support) a preformed oligonucleotide is bonded to
the support at or near one end. In this embodiment the
nature of the solid phase support and the method of
linkage between support and "starting" oligonucleotide are
25 a matter of choice, readily achievable by one skilled in
the art, using procedures known in the art with the
qualification that the structure of the solid phase must
not sterically hinder the assembly of the gene and the
linkage of the oligonucleotide to the support must
30 withstand the conditions of stepwise annealing, used for
subsequent gene assembly. The solid phase support for
this embodiment of step (1) appropriately comprises
nonporous latex microspheres derivatized by functional
groups, such that chemical crosslinking or condensation
35 can occur between the beads and a reactive group on one
end of the starting oligonucleotide in the assembly.

Again, the linkage of oligonucleotide to the latex particles can be achieved by a variety of established procedures which would be apparent to one skilled in the art. For example, hydrazide-derivatized latex particles are readily linked to oligonucleotides derivatized at the 5'-end with aldehyde or carboxylic acid groups as described by Kremsky et al., Nucleic Acids Res., 15:2891-2909, (1987)). Alternatively, alkylamine-derivatized beads may be linked to alkylamine-derivatized oligonucleotides, using a bifunctional crosslinking reagent such as disuccinimidyl suberate as described by Pilch & Czech, J. Biol. Chem. 254:3375-3381 (1979). In addition, alkylamine-derivatized latex particles can first be linked to avidin or streptavidin by glutaraldehyde activation such as described by Goodfried et al., Science 144:1344 (1964), then the first oligonucleotide in the assembly, labeled with biotin at its 5'-end, will attach to the beads through the well known tight avidin-biotin affinity.

The determination of whether the 5' or 3' end of the starting oligonucleotide (5' or 3') is attached to the solid phase support (which dictates the directionality of the gene assembly) is entirely a matter of choice, except when the linkage of starting oligonucleotide to the support is more conveniently achieved at one particular end of the oligonucleotide.

Step (2) in the gene assembly process (annealing of the next oligonucleotide in the desired gene or gene fragment) may be carried out under any of the standard annealing conditions known to those skilled in the art, for example, incubation at 50-65° C in the presence of 0.2-1 M NaCl or KCl, or incubation at 37° C in the presence of 0.2-1 M salt plus 50% formamide. The base sequence of the oligonucleotides may be chosen to satisfy the following specifications: (1) The desired base sequence of gene is generated by the assembly process; (2)

1 The extent of complementary overlap (yielding a duplex
segment holding the added oligonucleotide to the
support-bound component) may be any length so long as to
provide the required stability of the association is
5 provided. In a preferred embodiment the complementary
overlap sequence will be at least about 10 bases and may
be up to 50 bases; (iii) The length of protruding
single-stranded "tail" after annealing is preferably at
10 least 10 bases (the length yielding stable base pairing
with the subsequently added oligonucleotide); (iv)
Oligonucleotide sequences are preferably chosen to avoid
secondary structure within the oligonucleotides
(intrastrand base pairing resulting in hairpins), which
may interfere with annealing of the added oligonucleotide
15 to the support-bound component; and (v) The sequences are
chosen to avoid the production of more than one annealing
product (through a multiplicity of base pairing
possibilities).

Step (3) in the gene assembly (washing away of
20 excess, unannealed added oligonucleotide) can conveniently
be achieved by flow of solvent past the solid phase
support, for example through a reaction chamber containing
porous members at both ends. Alternatively, step (3) can
be accomplished by a series of brief centrifugation/
25 decanting steps in microcentrifuge tubes.

In step (4) (ordered, stepwise repeat of steps
(2) and (3) to build up the desired gene or gene fragment)
of one embodiment of the present invention, the
oligonucleotides assembled are designed to yield a
30 completely duplex DNA with strand interruptions at
positions alternating along both strands. In another
embodiment, the oligonucleotides are designed for assembly
of a partially duplex DNA molecule, in which
single-stranded gaps exist in alternating positions along
35 both strands. These gaps may be filled in by action of a
DNA polymerase in vitro.

1 Step (4) may also be carried out by addition of
several (eg., about 2-5) oligonucleotides in each
annealing step. Although this procedure potentially
reduces the total number of annealing steps required for
5 assembly of the desired gene or gene fragment, care must
be taken to insure that multiple products of annealing are
not generated, i.e., that all support-bound assemblies
generate the identical, desired duplex DNA sequence.

10 Step (5) (release of the assembled gene from the
support) is carried out by means chosen to be compatible
with the nature of the linkage of DNA to the support and
the structure of the assembled DNA. In one preferred
embodiment the stepwise annealing is carried out with all
15 oligonucleotides being 5'-phosphorylated except for one,
such that a completely duplex DNA is formed in which all
strand interruptions can be sealed by use of DNA ligase,
except for a single nonligatable strand interruption
adjacent to the support-linked oligonucleotide. Then the
contiguous duplex segment may then be removed from the
20 support by brief heating to 80-100° C. Alternatively, the
nonligatable strand interruption adjacent to the support
can be made by leaving a gap of one or more nucleoside
residues at this position in the assembled DNA.

25 In step 5 of another preferred embodiment,
appropriate oligonucleotides are selected for the assembly
such that a duplex DNA segment containing a restriction
enzyme recognition sequence is generated between the gene
or gene fragment and the support, such that release of the
DNA from the support can be conveniently achieved via
30 cleavage by the restriction endonuclease.

In all embodiments of the gene assembly process,
the DNA released from the support is conveniently cloned
into a vector for expression in cells and DNA sequence
analysis.

35 In accordance with another aspect of the present
invention, there is provided a process for assembly of

1 polypeptides, comprising the following steps: (1) attachment of a peptide to a solid phase support material; (2) stepwise end-to-end block condensation or ligation of peptides to the initial support-bound peptide, alternating
5 with washing steps, to construct a longer polypeptide, and (3) cleavage of the polypeptide from the support.

In a particularly preferred embodiment, the solid phase support comprises small diameter (5-50 micrometers) nonporous glass beads to which the first amino acid residue is covalently attached via a long chain alkylamine spacer arm. In another preferred embodiment, the solid phase support comprises small diameter (5-50 micrometers) glass beads containing pores of large diameter (1000-5000 Å). Both supports serve to avoid steric hindrance during
10 the assembly of long polypeptides. The peptide can be attached to the supports after the synthesis of the peptide, or alternatively, the glass beads can be first derivatized with an amino acid residue, then used for solid phase peptide synthesis to create a support-bound
15 peptide which is subsequently elongated in the assembly process. Although the preceding embodiments give examples of the kind of solid phase support and the type of linkage of peptide to the support which may be utilized, these parameters are a matter of choice. One skilled in the art
20 could devise alternate peptide-linked supports that possess the favorable steric properties suitable for polypeptide assembly. Peptide-linked nonporous latex microspheres may also be used as a solid phase support.
25

In step (2) of one preferred embodiment of the
30 solid phase polypeptide assembly (stepwise block condensation) of the present invention, the stepwise condensation of amino terminus-protected peptides onto the free amino terminus of a peptide linked to the support via its carboxy terminus, is carried out using the standard
35 Fmoc procedure. In another embodiment, stepwise block condensation on the solid phase support is performed

1 chemically, by use of a peptide bond-forming reagent such
as dichlorophenol, or enzymatically, by "reverse
proteolysis" (Offord, Protein Engineering, 1:151-157,
(1987)).

5 In accordance with still another aspect of the
present invention, there is provide a general procedure
for remodeling of biopolymer sequences on a solid phase
support, comprising the following steps: (1) attachment
10 of a high molecular weight biopolymer at one or more
positions in the biopolymer sequence to a solid phase
support; (2) excision of a specific segment of the
biopolymer; (3) washing away of the cleaving agents and
excised biopolymer segment; (4) addition of a chemically
synthesized biopolymer sequence or a fragment isolated
15 from natural sources and specific insertion of the added
segment into the biopolymer sequence to replace the
excised segment; (5) washing away of excess added
biopolymer segment and bond-reforming agents; and (6)
cleavage of remodeled biopolymer from the support. The
20 foregoing general procedure for biopolymer remodeling can
also be used to insert or delete biopolymer segments at
specific positions in the biopolymer sequence.

In step (1) of biopolymer remodeling the nature
of the solid phase support and means for its attachment to
25 the support are a matter of choice, depending on the
structure of biopolymer, and would be readily chosen from
existing applications by one skilled in the art. For
example, avidin-coated beads could be used to tightly bind
biotin-labeled DNA or biotin-labeled protein.
30 Alternatively, a specific antibody-bound support could be
used to bind an epitope in a protein or nucleic acid.
Also, a support-linked oligonucleotide (preferably 20-50
residues in length) could be used to link a
single-stranded DNA molecule to the support, via hydrogen
35 bonded base pairing. In addition, a reversible
crosslinking agent could be used to connect chemically
reactive groups in the biopolymer and support.

1 Site-specific cleavage of the biopolymer (step
2 of solid phase biopolymer remodeling) is preferably
achieved by enzymatic means, utilizing one or more
restriction endonucleases in the case of DNA, or specific
5 endopeptidases in the case of protein. In the specific
case of single-stranded DNA attached to the support,
cleavage by restriction endonuclease can be achieved by
adding oligonucleotides which anneal to the DNA to provide
short duplex regions containing the enzyme's recognition
10 sequence. Also, a specific chemical cleavage means (for
example, cleavage of protein by cyanogen bromide) can also
be employed in step (2).

In step (3) of solid phase biopolymer remodeling,
the cleaving agents and excised biopolymer segment are
15 washed from the support, preferably by flow of solvent
past the support-bound biopolymer contained within a
chamber fitted with porous means at both ends.
Alternatively, repeated brief centrifugation/decantation
steps can be used in step (3) for support-bound biopolymer
20 contained within microcentrifuge tubes.

In step (4) of solid phase biopolymer remodeling,
a "replacement" biopolymer segment is added, preferably in
molar excess over support-bound biopolymer, along with an
appropriate bond-reforming agent, to achieve replacement
25 of the biopolymer segment excised in step (2) by the
segment added in step (4). For example, in remodeling of
DNA, a restriction fragment isolated from natural sources
or a chemically synthesized duplex segment containing the
appropriate termini may be added, and ligated into the
30 position previously occupied by the segment excised in
step (2), by the action of DNA ligase. In the case of
protein, replacement of the excised segment by an added
peptide can be achieved enzymatically, by "reverse
35 proteolysis" catalyzed by specific endopeptidases under
reaction conditions such as disclosed in Offord, Protein
Engineering, 1:151-157, (1987)), or can be achieved

1 chemically, by action of a peptide bond-forming agent such
as dichlorophenol.

5 Washing away of excess reaction components from
the support-bound biopolymer (step (5)) may be achieved by
the same means as in step (3).

10 Cleavage of the remodeled biopolymer from the
support (step (6)) can be carried out by a variety of
means that would be apparent to one skilled in the art,
the method of choice depending on the nature of the solid
15 phase support and biopolymer and the type of linkage
between them. For example, for biopolymers attached to
the support via the avidin:SS-biotin affinity, the linkage
is readily broken by addition of buffer containing 100 mM
dithiothreitol Shimkus et al., Proc. Natl. Acad. Sci.

15 (USA) 82:2593-2597 (1985), dissociation of remodeled
biopolymer from an antibody affinity support can be
achieved by common protein denaturants, and release of a
DNA molecule base paired to a support-bound
oligonucleotide can be achieved by brief heating to

20 80-100° C.

Further objects, features and advantages of the
present invention will become apparent from a review of
the detailed description of the preferred embodiments
which follows, in view of the drawings, a brief
25 description of which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the solid
30 phase biopolymer assembly process as applied to
construction of a gene or gene fragment.

Figure 2 is a schematic diagram of the solid
phase biopolymer assembly process as applied to
construction of a polypeptide.

35 Figure 3 is a schematic diagram of the general
procedure for remodeling of a biopolymer on a solid phase
support.

1

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention will be described in detail by reference to the drawings. Although the embodiments described herein refer to a few specific uses of the present invention, many variations of the process are also within the scope of the present invention which will be obvious to one skilled in the art.

Figure 1 illustrates the concept of assembly of a gene or gene fragment on a solid phase support. A "starting" oligonucleotide 3 is first attached to a solid phase support 1. As discussed previously, the precise nature of the support 1 and the type of linkage 2 between the starting oligonucleotide and the support are a matter of choice, and are readily known to those of skill in the art. It is essential, however, that the geometry of the solid phase support is such that assembly of the gene is not sterically hindered, as it would be with most currently used solid phase support materials. To satisfy this requirement, a solid phase support consisting of small diameter (5-50 micrometers) nonporous glass beads, or alternatively, macroporous beads (5-50 micrometers in diameter) with very large pores (1000-5000 Å) are recommended for use in solid phase assembly of genes.

The linkage 2 of the starting oligonucleotide to the beads can take a variety of forms, readily known to those of skill in the art. The following examples of suitable linkages are given for illustrative purposes, and it is emphasized that alternative linkages, readily apparent to one skilled in the art, are also within the scope of the present invention.

One suitable linkage 2 to glass beads is the urethane linkage described by Sproat and Brown supra and incorporated herein by reference. The urethane linkage is ideally suited for a synthesis of a starting oligonucleotide of any base sequence prior to gene

1 assembly, since the urethane linkage is more stable than
the acyl linkages protecting the exocyclic amino groups of
A, G and C, such that the oligonucleotide will be retained
on the support under the conditions used for deblocking
5 the bases.

If the starting oligonucleotide in gene assembly
is designed to contain a sequence of I (inosine) and T
(thymidine) nucleosides, then the standard 3"-O-succinyl
linkage can be used to synthesize the starting
10 oligonucleotide, because an alkaline base-deblocking step
(which would hydrolyze the 3'-O-succinyl linkage) would
not be required after synthesis of oligo(I,T).

Several procedures are available for linkage of a
presynthesized starting oligonucleotide to the surface of
15 solid latex microspheres, providing a support-bound
oligonucleotide suitable for gene assembly. For example,
the well-known tight avidin-biotin affinity may be
employed, by covalently linking avidin to small
alkylamine-derivatized latex beads (0.1-10 microns in
20 diameter) by the glutaraldehyde activation or other
methods known in the art, producing avidin-coated beads
that will bind a 5'-biotin-labeled oligonucleotide.

Latex microspheres may also be covalently
attached to the starting oligonucleotide for gene assembly
25 by other methods, including the use of a homobifunctional
crosslinking agent such as disuccinimidyl suberate to link
alkylamine-derivatized latex beads with
5"-alkylamine-derivatized oligonucleotide, linkage of
hydrazide-derivatized latex beads to a
30 5"-aldehyde-oligonucleotide or to a
5'-carboxylate-oligonucleotide and other such linkage
methods are known in the art (Kremsky *et al.*, Sproat and
Brown, Shimkus *et al.*, Pilch and Czech, and Goodfriend *et*
al., *supra*, all incorporated herein by reference.

35 The protocol for solid phase gene assembly
illustrated in Fig. 1 calls for performance of a series of

1 stepwise annealings, using oligonucleotides 4, 5, 6, 7,
and n to build up the desired gene or gene
fragment. The degree of base "overlap" at each annealing
step will preferably result in formation of at least
5 twenty base pairs between added oligonucleotide and
support-bound single-stranded "tail." In the example
shown in Fig. 1, the starting oligonucleotide is attached
to the support via its 3"-end, and is
non-phosphorylated. Oligonucleotides added in the
10 stepwise annealing reactions are 5"-phosphorylated and
designed to form a fully double-stranded assembled DNA
(containing no single-stranded "gaps"), in which the
strand interruptions (5'-phosphate adjacent to 3'-OH) in
one strand are located at approximately the midpoint of
15 the oligonucleotides comprising the other strand. Under
these conditions, the "nicks" can be enzymatically
sealed by action of DNA ligase, prior to release of the
assembled gene from the support.

The stepwise annealing in gene assembly is
20 preferably carried out in a small volume (eg., 0.02-0.10
ml), with the solid phase support kept in suspension by
gentle agitation (except with submicron latex particles,
which are kept in suspension by Brownian motion). The
quantity of starting oligonucleotide attached to the
25 support can vary widely, for example, 0.01-1.0 micromoles
per gram of beads. At such a "loading capacity" of the
beads, essentially quantitative stepwise annealing would
occur within a few minutes under the following reaction
conditions (0.10 ml annealing volume): 50 mM potassium or
30 sodium phosphate, pH 7.5, 400 mM KCl or NaCl, 0.1-1.0
nanomole of support-bound oligonucleotide, 0.2-2.0
nanomole of added oligonucleotide, 50-60° C.
Alternatively, an identical reaction mixture, containing
addition of 50% formamide, could be incubated at 37° C.
35 Under the foregoing conditions the concentration of
annealing DNA (assuming 20 base pair overlap) is 20-200

1 micrograms per ml. The quantity of each added
oligonucleotide in the gene assembly is very low,
accommodating the use of inexpensive methods of
oligonucleotide synthesis that provide low yields of
5 purified product.

The stepwise annealing of one oligonucleotide at
a time is recommended, to insure that annealing occurs
specifically and quantitatively. However, it is possible
that the procedure illustrated in Fig. 1 could be
10 successfully adapted to the addition of several
oligonucleotides at a time, thereby requiring fewer steps
to assemble a gene. However, even with individual
annealings, a 1000 base pair gene could be assembled
within six hours, assuming assembly of fifty 40mers, five
15 minute annealing time and two minute washing time.

The washing step carried out after each annealing
reaction, which removes excess unannealed
oligonucleotides, thus assuring formation of the desired
annealing product in each cycle, is preferably carried out
20 by flow of solvent (eg., annealing buffer) past the
support, which may be provided for by housing the solid
phase support within a reaction chamber having porous
means at both ends such as that disclosed in U.S. Patent
application Serial No. 000,716.

25 Alternatively, 2-3 brief centrifugation/
decantation steps may be carried out (with support held
within a microcentrifuge tube) to achieve satisfactory
washing.

Obviously, in order to obtain the correct
30 assembly and structure of a gene it is critical that the
oligonucleotides added at each step be homogeneous.

After the completion of the gene assembly the DNA
product must be released from the support. In the example
shown in Fig. 1, this is simply achieved by a brief
35 heating step (80-90° C), which denatures the short duplex
section holding the assembled gene to the support, without

1 causing complete denaturation of the long assembled duplex
DNA (the latter having been converted to contiguous long
strands by action of DNA ligase). The unsealed strand
interruption at the beginning of the assembled gene (at
5 the junction of oligonucleotides 3 and 5 in Fig. 1,
resulting from the absence of a 5'-phosphate on the
support-bound starting oligonucleotide) could also be
arranged by formation of a nonligatable "gap" of at least
one base at this position.

10 Alternatively, the assembled gene or gene
fragment could conveniently be released from the support
by action of a restriction endonuclease, provided that its
recognition sequence were designed into the duplex DNA
near the support (e.g., within the duplex segment formed by
15 oligonucleotide 4 in Fig. 1). In the latter case,
5'-phosphorylation of the oligonucleotides used to
assemble the gene may be optional.

20 As illustrated in Fig. 1, the released gene (or
gene fragment) 9 may be used for any purpose, i.e., it may
be subsequently cloned into a vector for sequence
analysis, expression (production of protein encoded by the
gene), etc.

25 To achieve maximal benefit from the gene
synthesis and/or assembly of the present invention, the
base sequence of the oligonucleotides to be assembled
should be carefully planned, with the following
considerations in mind: (1) The sequences should be
designed to avoid formation of hairpins of four or more
base pairs within the oligonucleotides, which may
30 interfere with efficient intermolecular base pairing
during the annealing steps. (2) Sequences that are
commonly associated with poor coupling efficiency during
the chemical synthesis (such as four or more consecutive G
residues) should be avoided. (3) Sequences that introduce
35 "rare codons" into a gene should be avoided, if possible,
if the aim is to achieve high levels of gene expression.

1 "Rare codons" are those nucleotide sequences which are
rarely found in nature and thus may not be properly
translated in some hosts. (4) Oligonucleotides may be
designed to generate unique restriction sites within the
5 assembled gene to facilitate subsequent manipulations by
recombinant DNA techniques. For example, if mutations are
found at intervals within a chemically synthesized gene,
the existence of unique restriction sites permits cleavage
of individual cloned genes with restriction endonucleases,
10 and recombination to form the desired mutant-free gene.

The length of a duplex DNA that may be assembled
in the manner illustrated in Fig. 1 ranges widely, from
less than a hundred base pairs up to thousands of base
pairs. Because labor-intensive purification and analysis
15 of intermediates in the gene assembly are avoided by use
of the solid phase approach, time and expense associated
with gene construction are greatly reduced by use of the
present invention. Furthermore, the high efficiency of
the process permits the use of very small quantities of
20 DNA, further reducing the cost of gene synthesis. An
average size gene may be synthesized, assembled and cloned
into an expression vector within a period of one week, at
a total cost for materials and labor of less than \$1,000
if the segmented DNA synthesis device disclosed and
25 claimed in U.S. Patent Application No. 000,716, filed Jan.
6, 1987 is used to synthesize the oligonucleotides at 50
nanomole scale, and then the present invention were used
to assemble the gene. The cost of preparing the same gene
by conventional methods would be \$20,000 to \$50,000 and
30 would typically require about two months work.

The present invention although exemplified as a
means for gene assembly, is equally applicable to
construction of other biopolymers, including polypeptides
and polysaccharides. The method of the present invention
35 used for polypeptide assembly is shown in Figure 2. A
protein molecule could be constructed by ordered, stepwise

1 chemical condensation between the free amino terminus of a
peptide linked to the support via its carboxy terminus and
successively added amino terminus-protected peptides,
5 using standard Fmoc chemical condensation known to those
of skill in the art.

The present invention may also be applied to
remodeling of a biopolymer, a multistep process which, if
carried out in solution by conventional means, frequently
requires time-consuming and labor-intensive purification
10 and analytical steps before the desired end product is
obtained. By performing the same manipulations on a solid
phase support rather than in solution, the need for
purification and analysis of intermediate products is
avoided, thus saving time and labor. The advantage of
15 carrying out a biopolymer reconstruction on a solid phase
support is illustrated in Figure 3.

Biopolymer 11 is first attached to a solid phase
support 1 at one or more positions in the biopolymer
sequence. As explained previously for the process of
20 biopolymer assembly, the precise nature of the solid phase
support and the method of linkage of biopolymer thereto
are entirely a matter of choice, the only constraint being
that the structure of the solid phase support must not
restrict accessibility of reaction components to the
25 biopolymer. Suitable solid phase supports, types of
linkages, means for washing away reaction components and
means for ultimate release of biopolymers from the
supports such as those described previously for biopolymer
assembly may be utilized.

30 The support-bound biopolymer 11 (for example, a
double-stranded plasmid DNA) is treated with at least one
agent (for example, restriction endonuclease(s)) to
produce cleavage at one or more specific sites 13 within
the biopolymer sequence. If the biopolymer is cleaved at
35 two specific sites, one or more specific fragment(s) 12
were released (for example, a restriction fragment). The

1 released fragment(s) 12 and cleaving agent(s) are
conveniently washed away, as described previously for
biopolymer assembly, then a replacement fragment 14 (for
example, a restriction fragment or synthetic duplex DNA)
5 is added, and the bonds are reformed (for example, by DNA
ligase), producing the remodeled biopolymer 15.

This procedure may be used to produce a deletion
within the biopolymer, by reforming the bonds after
removal of the released segment 12, without adding back a
10 replacement segment.

In addition, this procedure may be used to insert
an additional biopolymer segment into a specific location
within the support-bound biopolymer, by cleaving at a
single site within the biopolymer, and then attaching a
15 biopolymer segment at this position (for example,
insertion of a "foreign" duplex segment or synthetic
duplex DNA at a unique restriction site within a cloning
vector, to produce a recombinant DNA).

The application of the present invention to
20 recombinant DNA technology is advantageous, because of the
elimination of time-consuming purification steps that are
typically carried out in order to remove a released DNA
segment before replacing it with another sequence.

25

EXAMPLE 1

Remodeling of Bacteriophage M13 Vector

A specific application of the present invention
for manipulation of single-stranded circular DNA (such as
a bacteriophage M13 vector) is now given, with reference
30 to Fig. 3. A circular, single-stranded phage DNA 11 is
conveniently attached to the solid phase support via base
pairing with a support-bound synthetic oligonucleotide
(20-50 bases in length, complementary to a specific region
within the single-stranded vector). Next, two synthetic
35 oligonucleotides (eg., 20mers) are added and allowed to
anneal with the vector sequence, producing short duplex

1 regions containing the restriction sites 13.

Restriction endonuclease(s) are then used to cleave out a segment 12 of the DNA between the restriction sites, and the restriction enzyme(s) and released fragment 5 are washed away. Then a replacement fragment 14 (containing short duplex regions at the ends, with identical termini as in the fragment removed) is added and joined to the support-bound DNA by action of DNA ligase. When the cleavage of support-bound single-stranded vector 10 is carried out at a single, unique position, a restriction fragment or synthetic DNA will be inserted at this position. After these manipulations are performed, the DNA can be made completely double-stranded by action of a DNA polymerase, and as polymerization proceeds through the 15 short duplex region connecting the vector to the support-bound oligonucleotide, the vector is released from the former, by the well-known "strand displacement" phenomenon. Finally, the DNA may be converted to closed circular form by action of DNA ligase.

20

EXAMPLE 2

Synthesis and assembly of a segment of the E. coli lacI gene.

Solid Phase Support:

25

The support used for both synthesis of the "starting" oligonucleotide and for subsequent gene assembly consisted of long chain alkylamine-derivatized solid glass beads of 6 micrometer diameter, derivatized with 5'-trityl,2'-deoxythymidine to form the 3'-O-urethane 30 linkage (Sproat and Brown, 1985). The "loading" capacity of the support, determined by HPLC analysis following release of nucleoside from support by 24 hour treatment at 55° C in concentrated ammonium hydroxide, was 2.2 micromoles nucleoside per gram of support.

35

1 Synthesis of Oligonucleotides:

45 mg of support was derivatized by the method described by Sproat and Brown with 0.1 umoles of a first nucleoside (A). A non-phosphorylated oligonucleotide
5 (3'-AAAAAAAAAAAAAGCGTCGCACGCT-5') was synthesized on the support by the phosphoramidite method (Beaucage & Caruthers, 1981), using a Milligen 7500 DNA synthesizer. Following the final detritylation step, the support material was placed into a glass vial and treated with 1.5
10 ml concentrated ammonium hydroxide at 55° C. for 1 hour to remove the protecting groups from the exocyclic amino groups. The support material was then placed into a 1.5 ml Eppendorf tube and washed five times (by centrifugation/decantation) with annealing buffer (as
15 specified below).

Annealing Conditions for Gene Assembly:

To a 1.5 ml Eppendorf tube was added 0.5 mg oligonucleotide-support (approx. 1 nmole), 2 nanomole of the 5'-phosphorylated oligonucleotide,
20

5'-pTTTCGCAGCGTGCAGCGTGCCGGGTGGT-3'

and sufficient annealing buffer (50 mM KH₂PO₄, pH 7.5, 400 mM KCl, 1mM EDTA) to bring the volume to 0.10 m. The
25 tube was incubated at 55° C. for 5 minutes, with occasional gentle agitation, then the tube was centrifuged for 1 minute in an Eppendorf microcentrifuge and the beads were washed twice with 1 ml annealing buffer.

After the first annealing and washing steps the
30 product is:

Sup-U-O-3'-AAAAAAAAAAAAAGCGTCGCACGCT-5'

5'-pTTTCGCAGCGTGCAGCGTGCCGGGTGGT

35 The annealing/washing cycle was repeated, using 2 nanomole each of the following 5'-phosphorylated oligonucleotides in succession:

1

3'-CGCACGGGCCACCACTGGTCCGGTCGGTp-5' (3)

5'-pGAACCAGGCCAGCCACGTTCTGCGAAAAC-3' (4)

3'-GCAAAGACGCTTTGAGCTp-5' (5)

5

The final product, the lacI gene fragment coupled to the solid support is depicted in Figure 4.

Ligation and Cleavage of Duplex DNA From the Support:

10 The support was washed twice with ligase buffer (50 mM Tris-HCl, pH 7.8, 20mM dithiothreitol, 10mM MgCl₂, 1mM ATP, 0.05 mg/ml bovine serum albumin), then resuspended in 0.098 ml ligase buffer. Two microliters of DNA ligase was added (New England Biolabs, high specific activity grade). After incubation for 30 minutes at 37° C., with occasional gentle agitation, the support was then washed twice with Ava I buffer (10mM Tris-HCl, pH 8, 50mM NaCl, 10mM MgCl₂, 5mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin), then resuspended in 0.098 ml of this buffer. Two microliters of restriction endonuclease Ava I (New England Biolabs) were added, and the mixture was incubated at 37° C. for 30 minutes to cleave the DNA from the support. The tube was centrifuged and the supernatant was collected. The beads were washed twice with 0.1 ml Ava I buffer, and the DNA in the combined supernatants were ethanol precipitated and dissolved in 0.1 ml of 10mM Tris-HCl, pH 7.5 containing 1mM EDTA.

Cloning and Analysis of the Synthetic Gene Segment:

30 Approx. 1 nmol of the duplex lacI gene fragment prepared above was mixed with 1 nmol of M13-laci-SAXB which had been previously cleaved with Ava I and passed over a Sepharose 2B column to remove the 40-bp segment of the lacI gene. The DNA was ligated with 4% DNA ligase as described above. The DNA was then transfected into E. coli strain JM107 and progeny phage were plated into E.

1 coli strain PD8. This genetic system provides the
opportunity to assess the possible generation of mutations
during the chemical synthesis of the lacI gene fragment
(mutations are seen as blue plaques in the absence of
5 inducer). The frequency of mutations in this experiment
was undetectable over the spontaneous frequency.

The DNA of the semi-synthetic M13-lacI was
sequenced by the "dideoxy" method and found to contain the
desired wild-type sequence in the region of the chemical
10 synthesis. Thus, the DNA duplex synthesized by the method
of the present invention was identical in both sequence
and mutation frequency to that of the naturally occurring
wild-type lacI sequence.

Although Fig. 2 illustrates a general procedure
15 for biopolymer reconstruction (remodeling), many
variations of the process, specific for different
biopolymers and different types of manipulations thereupon,
will be evident to one skilled in the art and are within
the scope of the present invention. For example, the
20 solid phase remodeling process could be used to replace a
specific segment of a protein with a different, modified
segment, a solid phase "recombinant protein" technique
analogous to the solid phase recombinant DNA example
discussed previously.

25 One skilled in the art will readily appreciate
that the present invention is well adapted to carry out
the objects and obtain the ends and advantages mentioned,
as well as those inherent therein. The components,
methods, procedures and techniques described herein are
30 presently representative of the preferred embodiments, are
intended to be exemplary, and are not intended as
limitations on the scope of the present invention.
Changes therein and other uses will occur to those skilled
in the art which are encompassed within the spirit of the
35 invention and are defined by the scope of the appended
claims.

WHAT IS CLAIMED IS:

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1 1. A process for assembling a biopolymer from oligomeric subcomponents thereof, comprising the steps of:

5 (1) attaching one end of a biopolymer oligomeric subcomponent to a solid phase support;

10 (2) attaching a next oligomeric sequence at or near the free end of the support-bound component;

15 (3) removing excess, unattached oligomeric sequences;

20 (4) repeating steps 2 and 3 for the ordered, stepwise attachment of oligomeric biopolymer subcomponents at or near the free end of the support-bound component resulting in assembly of the biopolymer; and

25 (5) releasing the assembled biopolymer from said support.

2 2. A process for assembling a gene from synthetic oligonucleotides comprising the steps of:

20 (1) attaching a first oligonucleotide to a solid phase support at or near a first end of said oligonucleotide;

25 (2) removing excess, unattached oligonucleotide;

30 (3) hybridizing a next oligonucleotide containing at least 5-100 base pairs complementary to the free end of said bound oligonucleotide wherein said next oligonucleotide contains at least 5-100 base pairs more than said complementary sequence;

35 (4) removing excess, unattached oligonucleotide;

35 (5) repeating steps (3) and (4), for the ordered, stepwise hybridization of oligonucleotide sequences at or near the free end of the support-bound component; and

1 (6) releasing the assembled gene or gene fragment from the support.

3. A process as claimed in claim 1, wherein
5 said biopolymer is selected from the group consisting of gene or portion thereof, a genome or portion thereof, a ribonucleic acid, a polypeptide, and a polysaccharide.

4. A process as claimed in claim 1, wherein the
10 successively added components become noncovalently attached to the support-bound biopolymer component by noncovalent forces, including hydrogen bonding, electrostatic interaction or hydrophobic interaction.

15 5. A process as claimed in claim 1, wherein the successively added components become covalently bonded to the support-bound biopolymer by means of chemical or enzymatic reaction.

20 6. A process as claimed in claim 1, wherein said solid phase support consists of a nonporous, particulate material selected from the group consisting of silica (glass), latex, polystyrene and plastic.

25 7. A process as claimed in claim 1, wherein said solid phase support consists of a macroporous material containing intramatrix spaces (pores) large enough to prevent steric hindrance of biopolymeric assembly.

30 8. A process for remodeling of a biopolymer partial substitution or modification, comprising:
 a solid phase support material;
 means for attachment of a biopolymer to said support material at one or more positions in the biopolymer sequence;

1 means for excision of at least one specific
segment of the support-bound biopolymer, the
excised segment being washed away, then replaced
by addition of a modified, replacement segment
5 which becomes specifically attached to the
support-bound biopolymer, thus providing for
segmental substitution of the biopolymer; and
means for removal of the remodeled
biopolymer from the support.

10 9. A process as claimed in claim 8, wherein the
support-bound biopolymer is a double-stranded DNA
molecule, the means for excision comprises a restriction
endonuclease, the replacement segment comprising a
15 double-stranded DNA segment, produced synthetically or
isolated from natural sources, and the means for rejoining
of the replacement segment to the support-bound DNA
comprising DNA ligase.

20 10. A process as claimed in claim 8, wherein the
support-bound biopolymer is a single-stranded DNA
molecule, the means for excision comprises addition of one
or more restriction endonucleases and one or more
synthetic oligonucleotides which anneal to the
25 single-stranded DNA to create cleavage sites for action of
the restriction endonuclease(s), the replacement segment
comprising a double-stranded DNA segment, produced
synthetically or isolated from natural sources, and the
means for rejoining of the replacement segment to the
30 support-bound DNA comprising DNA ligase.

11. A process as claimed in claim 8, wherein the
support-bound biopolymer is a polypeptide and the means
for excision comprises a specific endopeptidase, the
replacement segment comprises a peptide, produced
35 synthetically or isolated from natural sources, and

1 wherein the means for linkage of the added segment to the support-bound polypeptide comprises chemical condensation or enzymatic ligation.

5 12. A process as claimed in claim 8, wherein rejoicing of the biopolymer is carried out without addition of a replacement segment, resulting in deletion of one or more segments from the biopolymer.

10

13. A process as claimed in claim 8, wherein cleavage of the biopolymer occurs at a single site, and insertion of added biopolymer segment occurs at this site.

15

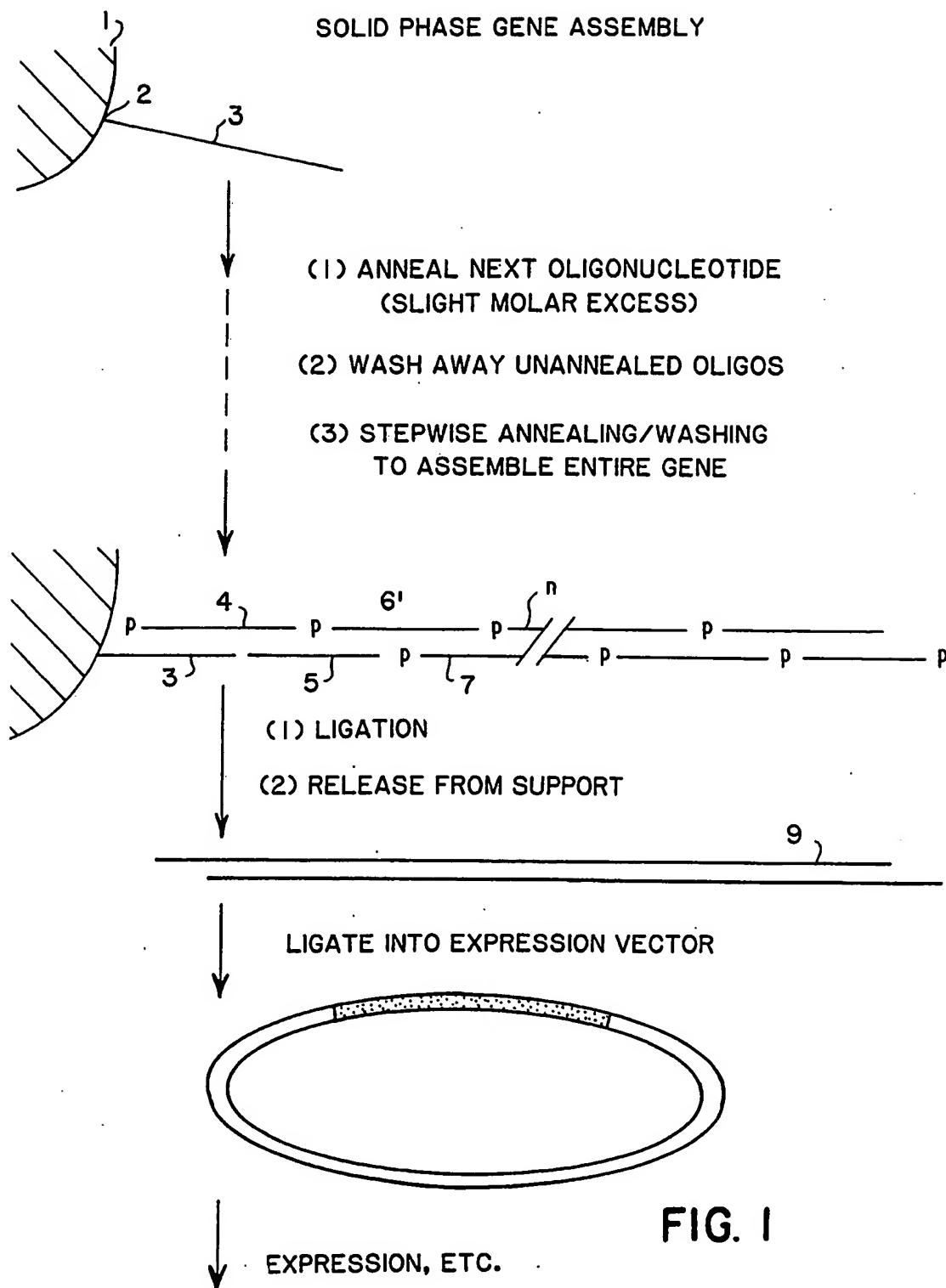
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SOLID PHASE POLYPEPTIDE ASSEMBLY

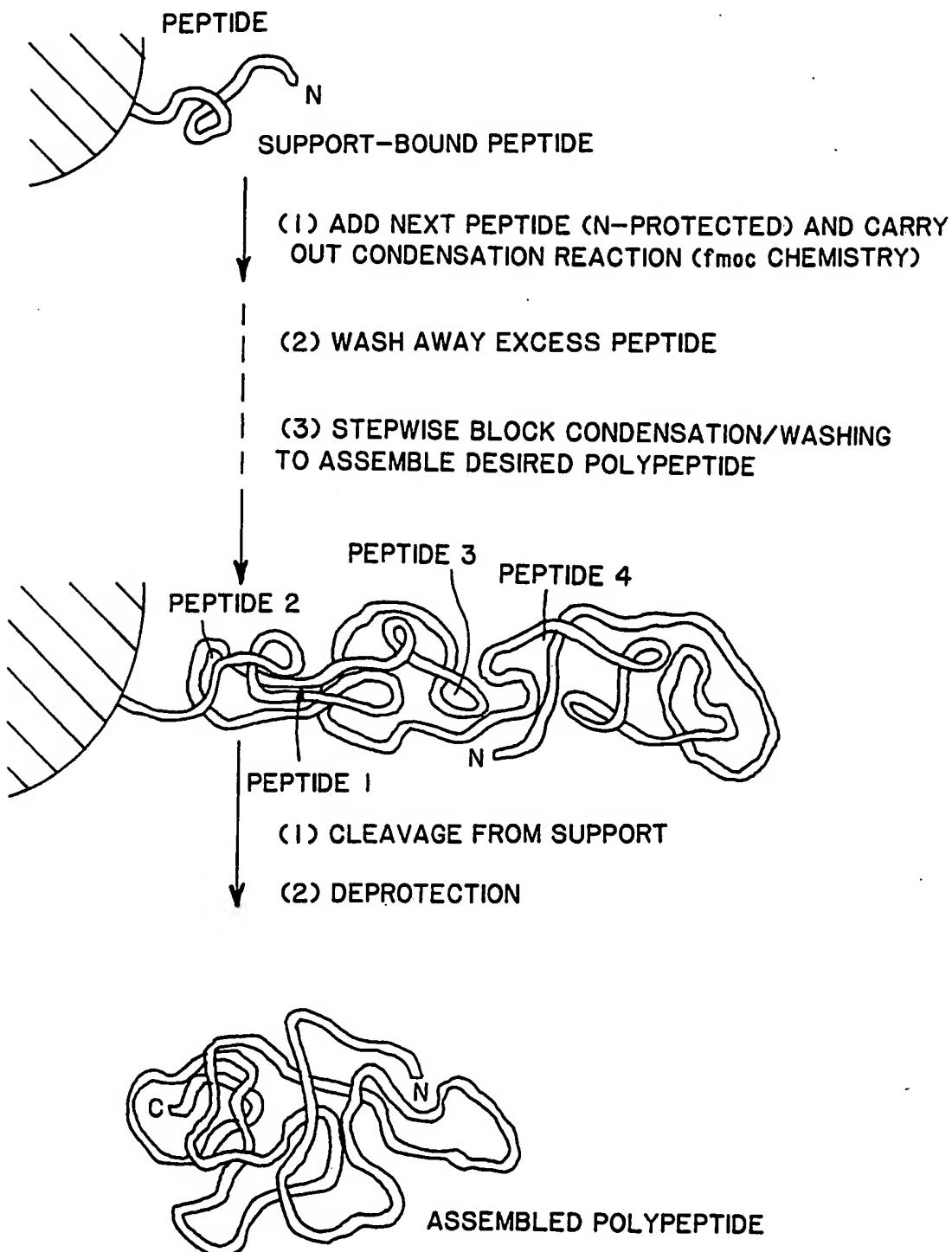


FIG. 2

SUBSTITUTE SHEET

3/4

SOLID PHASE REMODELING OF A BIOPOLYMER

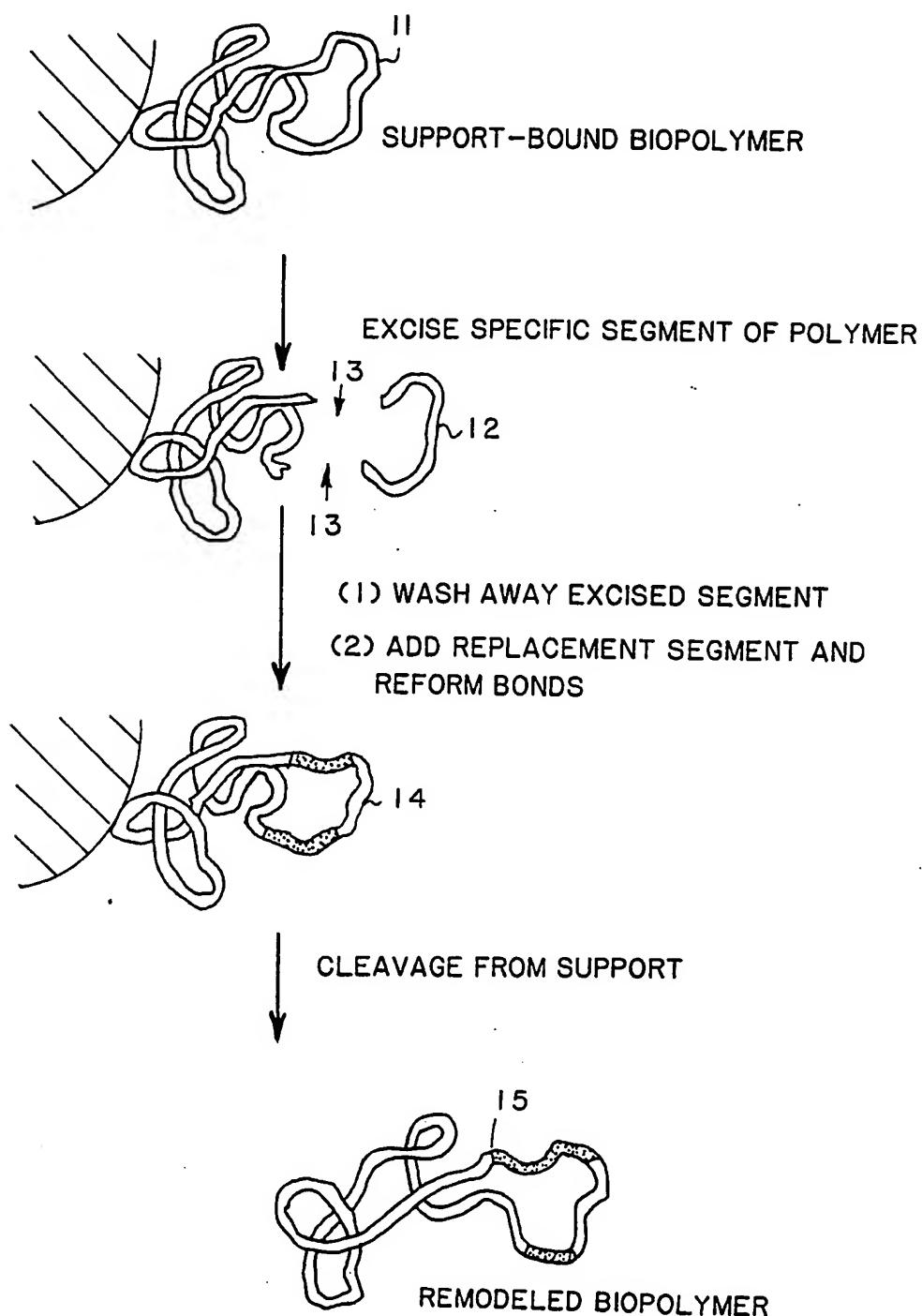
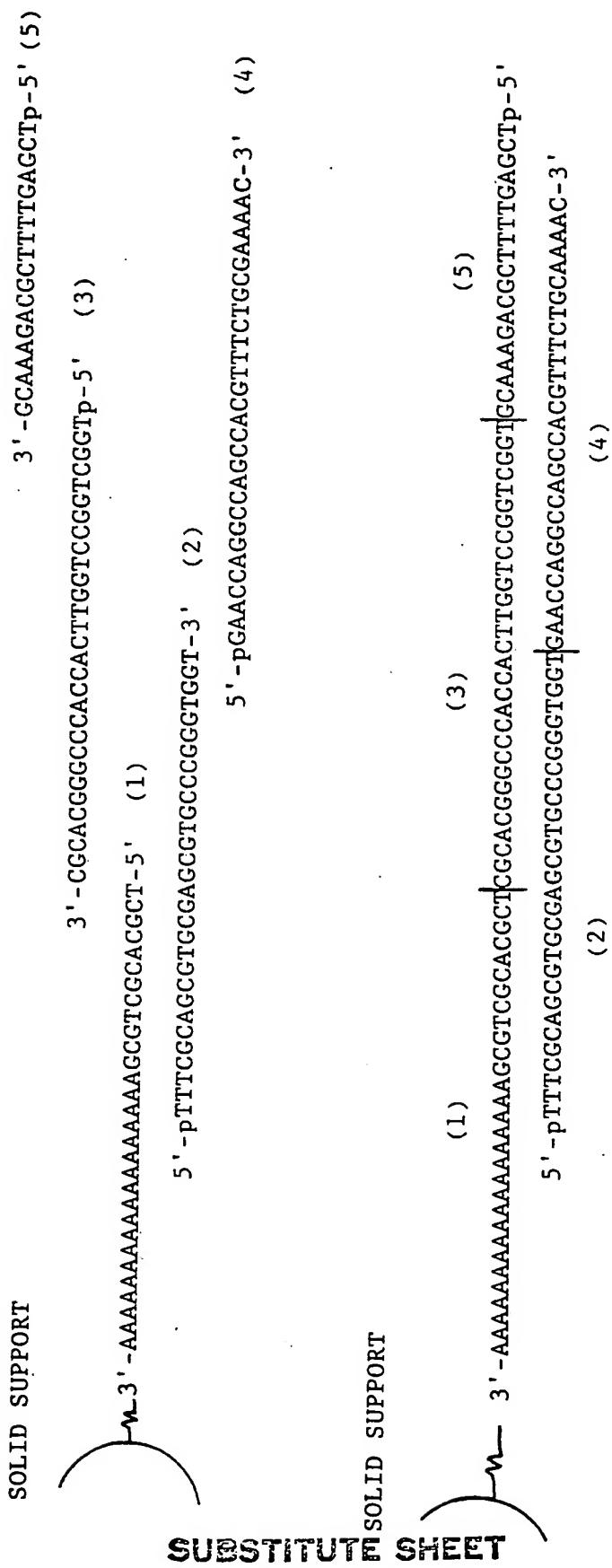


FIG. 3
SUBSTITUTE SHEET

4/4

**FIG. 4**

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02915

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) only both National Classification and IPC
 IPC(4): C12Q 01/68; C12P 21/00; C12P 19/34; G01N 33/00; C07K 1/04
 U.S.C1.: 435/6; 435/68; 435/91; 436/86; 436/94; 530/334;
 SEE ATTACHMENT

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/ 6, 68, 91, 172.5
	436/ 86, 94
	530/ 334
	536/ 27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

CHEMICAL ABSTRACT DATA BASE (CA): 1967-1989; BIOSIS DATA BASE: 1969-1989; KEYWORDS: solid phase, gene, oligonucleotide, assembly, synthesis, protein, biopolymer, polypeptide, polysaccharide, muta?

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	NUCLEIC ACIDS RESEARCH, Volume 15, issued 1987, August, (HOSTOMSKY ET AL.), "Solid-Phase Assembly of Cow Colostrum Trypsin Inhibitor Gene", See pages 4849-4856 particularly the abstract.	I-7
Y	ANNUAL REVIEW OF GENETICS, Volume 15, issued 1981, November, (D. SHORTLE), "Directed Mutagenesis", See pages 265-294 particularly pages 266, 270 and 271	8-10 and 12-13
X	JOURNAL OF ORGANIC CHEMISTRY, Volume 48, issued 1983, March, (NAKAGAWA ET AL.), "Polymer-Bound Oxime: Application to the Synthesis of a Peptide Model for Plasma Apolipoprotein A-I", See pages 678-685 particularly the abstract.	1-8 and 11
X	CHEMICAL ABSTRACTS, Volume 107, no. 21, issued 1987, November, (LYLE ET AL.), "Chemical Synthesis of Rat Atrial Natriuretic Factor by Fragment Assembly on a Solid Support", See page 222, column 1, the abstract no. 198913u, J. Org. Chem., 1987, 52(17), 3752-9 (Eng.).	1-8 and 11

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

11 SEPTEMBER 1989

9/6/89

International Searching Authority

ISA/US

Signature of Authorized Officer

Richard C. Peet
RICHARD C. PEET

PCT/US89/02915

I. CLASSIFICATION AND SUBJECT MATTER (CONTINUED)

IPC(4): C12N 15/00; C07H 15/12

U.S.C1.: 435/172.3; 536/27

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